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Antimicrobial susceptibility of anaerobic bacteria

In vitro emergence of fluoroquinolone resistance in *Cutibacterium* (formerly *Propionibacterium*) *acnes* and molecular characterization of mutations in the *gyrA* gene

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ABSTRACT

In vitro occurrence of levofloxacin (LVX) resistance in *C. acnes* and characterization of its molecular background were investigated. The mutation frequency was determined by inoculation of 10^8 cfu of *C. acnes* ATCC 11827 (LVX MIC = 0.25 mg/L) on LVX-containing agar plates. The progressive emergence of resistance was studied by a second exposure to increasing LVX concentrations. For mutants, the QRDR regions including the *gyrA* and *parC* genes were sequenced and compared to both *C. acnes* ATCC 11827 and *C. acnes* KPA171202 reference sequences (NC006085). The importance of the efflux pump system in resistance was investigated by using inhibitors on selected resistant mutants with no mutation in the QRDR. *C. acnes* growth was observed on LVX-containing plates with mutation frequencies of 3. 8 cfu $\times$ 10^{-8} (8 $\times$ MIC) and 1.6 cfu $\times$ 10^{-7} (4 $\times$ MIC). LVX resistance emerged progressively after one-step or two-step assays. In LVX-resistant isolates, the MIC ranged from 0.75 to >32 mg/L. Mutations were detected exclusively in the *gyrA* gene. Ten genotypes were identified: G99 C, G99 D, D100N, D100 H, D100 G, S101L, S101W, A102 P, D105 H and A105 G. Mutants S101L and S101W were always associated with a high level of resistance. Mutants with no mutation in the QRDR were more susceptible when incubated with an efflux pump inhibitor (phenyl-arginine β-naphthylamide) only, suggesting, for the first time, the expression of such a system in *C. acnes* LVX-resistant mutants.

1. Introduction

Cutibacterium acnes is clearly involved in the skin disorder acne [1,2]. Since the 1970s, in the dermatology field, resistant *C. acnes* strains have been reported with different mechanisms being described, particularly in macrolide and tetracycline resistance [3]. *C. acnes* is also a recognized cause of foreign-body infections, including prosthetic joints (PJI), spine-hardware and ventriculo-peritoneal shunts [4,5]. Interestingly, under these circumstances, *C. acnes* remains highly susceptible to a wide range of antimicrobials. However, the optimal treatment regimen of *C. acnes* biofilm infections has not yet been defined [6]. The effectiveness of rifampicin in eradicating *C. acnes* biofilms has been demonstrated *in vitro* [7] and recently *in vivo* in an animal model of foreign-body infection [8].

Rifampicin is a key antibiotic in PJI [6]. The emergence of resistance has recently been reported *in vitro* (selected mutants) [9] and in clinical strains involved in device-related or biofilm infections [10]. Rifampicin resistance has also been described in other *Cutibacterium* species [11,12]. To avoid the emergence of a resistant strain, it is always recommended to use rifampicin in combination with other drugs, especially fluoroquinolones (FQ) [8]. The addition of levofloxacin prevents the selection of rifampicin-resistant mutants [9]. In addition, different studies have reported FQ-resistant *C. acnes* strains [13–16] but only five clinical strains have been explored at the molecular level [17]. As in other Gram-positive bacteria, FQ resistance in *C. acnes* strains appears to be due to

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mutations in the DNA gyrase and topoisomerase IV genes (*gyr*A and *par*C, respectively) [18]. The alterations usually occur in the Quinolone-Resistance-Determining Region (QRDR). According to the classic *Escherichia coli* numbering, the most frequently described mutations are found at positions 83 and 87 in GyrA and 80 and 84 in ParC [19].

The aims of this study were (i) to investigate the possible *in vitro* emergence of levofloxacin resistance in *C. acnes,* (ii) to assess the impact of different mutations in the QRDR responsible for resistance after *in vitro* levofloxacin selection, and to characterize the molecular background of these mutants. In fact, susceptible clinical strains involved in PJI present a levofloxacin MIC of 0.25 mg/L and, despite some descriptions in the literature, little information is available to explain fluoroquinolone resistance in *C. acnes*.

2. Materials and methods

2.1. Studied organism

All experiments were performed with the *C. acnes* reference strain ATCC 11827. The MICs of ofloxacin, ciprofloxacin, levofloxacin and moxifloxacin were 0.19, 0.25, 0.25 and 0.064 mg/L, respectively. Bacteria were stored at −70 °C using the cryovial bead preservation system (Microbank; Pro-Lab Diagnostics, Richmond Hill, Ontario, Canada). For inoculum preparation, one bead was spread on a blood agar plate and incubated for 72 h at 37 °C. After growth, one distinct colony was suspended in 10 mL of reduced brain heart infusion (Becton-Dickinson, Le Pont de Claix, France) and incubated at 37 °C for another 72 h. All incubations were performed in anaerobic conditions using an AnaeroGen™ system (Oxoid, Basingstoke, Hampshire, UK) at 37 °C. Fifteen levofloxacin-susceptible clinical *C. acnes* isolates were also investigated as a control.

2.2. Identification and susceptibility of clinical strains

All isolates were identified by matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry with a VitekMS® mass spectrometer (bioMérieux SA, Marcy-l'Etoile, France) as previously reported [20]. *In vitro* susceptibility was tested using the disk diffusion method with Brucella agar plates under anaerobic conditions, and the results were interpreted according to CA-SFM 2013 guidelines (http://www.sfm-microbiologie.org/UserFiles/files/casfm/CASFM2013vjuin.pdf). Resistance to ofloxacin, ciprofloxacin, levofloxacin and moxifloxacin was confirmed by the Etest method (bioMérieux, Marcy-l'Etoile, France).

2.3. Levofloxacin resistance

2.3.1. Single-step levofloxacin-resistant mutant

For the analysis of spontaneous levofloxacin resistance, agar-based single-step mutation studies were performed, as previously described [9]. Brucella agar supplemented with vitamin K, hemin and horse blood, prepared according to CLSI M11-A7 guidelines, was used [21]. Briefly, a large inoculum (10^8 cfu) of the bacteria was spread on Brucella agar plates containing levofloxacin concentrations of 1 × to 128 × MIC, corresponding to 0.25–32 mg/L, respectively. Since there are no breakpoints for anaerobes, the mutants were classified into two categories, one with a low resistance level (levofloxacin MIC = 0.5 mg/L to 6 mg/L) and the other with a high resistance level (levofloxacin MIC>6 mg/L). The plates were incubated for 7 days before the colonies were enumerated. The spontaneous resistance frequency was calculated from the number of colonies grown on plates containing drug *versus* the number of colonies grown on drug-free agar. To investigate whether a secondary agent could prevent the emergence of levofloxacin resistance, experiments were performed as described above in the presence of 0.5 × MIC of rifampicin (MIC = 0.007 mg/L) or amoxicillin (0.064 mg/L).

2.3.2. Two-step levofloxacin-resistant mutant

Colonies growing at the lowest antibiotic concentration (>0.5–6 mg/L, considered a low level of resistance) were sampled, checked for purity, grown overnight in antibiotic-free broth, and plated again on new antibiotic gradient plates. Bacteria were exposed to a maximum of two consecutive passages on the antibiotic gradients. MICs were determined after both passages.

The stability of all levofloxacin-resistant mutants was confirmed by sub-culturing on levofloxacin-free agar three times. The levofloxacin MIC was retested by Etest using an inoculum of 1 McFarland as recommended by CA-SFM 2013 and anaerobic incubation for 48 h at 37 °C. All resistant mutants were identified as previously described.

2.3.3. Antimicrobial agents

Levofloxacin solution (5 mg/mL) for the selection of resistant mutants was purchased from Sanofi Aventis Pharma AG, France.

2.4. Detection of mutations in both gyrA and parC genes in levofloxacin-resistant C. acnes mutants

Total DNA from *C. acnes* ATCC 11827 and isogenic resistant mutants was extracted using the InstaGene Matrix method (Bio-Rad Laboratories, Hercules, CA, USA) according to the manufacturer's instructions. After centrifugation, the supernatant was used as the DNA template for PCR analysis.

The partial *gyrA* and *parC* genes, including the QRDR, were amplified by PCR. Different sets of primers were designed according to the sequence alignment of four strains (GenBank accession numbers) and are presented in Table 1. Two regions of the *gyrA* and *parC* genes from *C. acnes* were amplified: a 454 bp fragment from nucleotide positions +11861 to +12297 for *gyrA* and a 477 bp fragment from nucleotide positions +1140659 to +1140201 for *parC* corresponding to the whole genome (*C. acnes* coordinates using *C. acnes* KPA171202; GenBank accession number NC006085) [22]. PCR was performed in a final volume of 50 μL containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM $MgCl_2$, 200 μM of each nucleotide, 0.5 μM of each primer and 2.5 U of *Taq* DNA polymerase (Phusion® High-Fidelity DNA Polymerase; Finnzymes, Illkirch, France). The PCR conditions were as follows: a 5 min first step of denaturation at 94 °C, 30 cycles with 60 s of denaturation at 94 °C, 60 s of hybridization at 55 °C and 60 s of extension at 72 °C, with a final extension step of 7 min at 72 °C.

The PCR fragments were purified and sequenced using the ABI PRISM BigDye Terminator v1.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Courtaboeuf, France). Sequence analysis was performed on a 3130XL Genetic Analyzer DNA sequencer (Applied Biosystems, Courtaboeuf, France). The sequences of both *gyrA* and *parC* genes were compared to that of *gyrA* and *parC* genes of the *C. acnes* reference strain (GenBank accession number NC006085) and the wild-type *C. acnes* ATCC 11827 strain using different free software available on the Internet (https://blast.ncbi.nlm.nih.gov/Blast.cgi, http://www.genome.jp/tools/clustalw/ and http://web.expasy.org/translate/). A comparison of the 104 whole genomes available on Pubmed was also carried out to look for previously described strains with point mutations in the QRDR among the strains that now comprise the Human Microbiome Project and other sequencing projects.